AD_____

Award Number: DAMD17-00-1-0313

TITLE: New Agents for Taxol-Resistant Breast Adenocarcinoma

PRINCIPAL INVESTIGATOR: Jim Klostergaard, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas M.D. Anderson Cancer Center
Houston, Texas 77030

REPORT DATE: July 2004

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2004	Final (1 Jul 2000 - 30 Jun 2004)

4. TITLE AND SUBTITLE
New Agents for Taxol-Resistant Breast Adenocarcinoma

5. FUNDING NUMBERS
DAMD17-00-1-0313

6. AUTHOR(S)
Jim Klostergaard, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas M.D. Anderson Cancer Center
Houston, Texas 77030

E-Mail: jkloster@mdanderson.org

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Taxol has proven to be very active in breast cancer; however, evidence for resistance to Taxol has emerged. One approach to overcoming drug resistance involves drug copolymers. The paclitaxel copolymer, PGA-TXL, has shown both reduced toxicity and greater tumor localization in animal models. It has also demonstrated reduced toxicity and greater ease of administration compared to Taxol in the clinic, and has shown activity in patients with Taxol-refractory tumors. MDA-MB-361 human breast adenocarcinoma cells were implanted orthotopically in the mammary fat pad of female nude mice. When tumor volumes reached ~20 mm^3, one group was treated with PGA-TXL, 180 mg/kg i.p. Two other groups received a multiple-dose regimen of either 5 or 10 mg/kg Taxol, i.p. Control tumor volumes increased 5.89 +/- 0.43-fold (mean +/- SEM) over the next 35 days. Treatment with PGA-TXL was highly efficacious: the increase in tumor volumes in this group was 2.96 +/- 0.31-fold during the same time period ($p = 0.0006$). The two Taxol-treated groups failed to demonstrate significant responses: tumor volumes increased 6.06 +/- 0.25 –fold ($p = 0.74$) and 4.29 +/- 0.61 -fold ($p = 0.101$) for the 5 and 10 mg/kg groups, respectively. The key results from this study indicates that even single-dose PGA-TXL is active against MDA-MB-361, an orthotopically-implanted human Her-2/neu over-expressing breast tumor model that is highly resistant to a multiple-dose regimen of Taxol. Our pre-clinical studies suggest that among the patients who could be considered for trials with PGA-TXL are those with tumors over-expressing HER-2/neu and refractory to conventional taxanes.

14. SUBJECT TERMS
PGA-TXL, HER-2/neu, drug resistance, orthotopic, human breast adenocarcinoma

15. NUMBER OF PAGES
43

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover.....1

SF 298.....2

Table of Contents.....3

Introduction.....4

Body.....5

Key Research Accomplishments.....8

Reportable Outcomes.....8

Conclusions.....13

References.....13

Appendices.....15

INTRODUCTION

Taxol has proven to be a valuable addition to the chemotherapeutic regimens that can be offered to breast cancer patients; however, as with other drugs, evidence for resistance to Taxol has emerged. Among these resistance mechanisms is the P-gp170 membrane-associated drug-efflux pump, for which the active agent, paclitaxel, is a substrate, and over-expression of the oncogene, HER-2/neu. Both of these resistance mechanisms are widely associated with breast cancer. Strategies to address Taxol-resistance include its combination with other chemotherapeutic agents and dose-intensification. However, in recent randomized clinical trials, the latter has proven to be largely ineffective, with little meaningful clinical benefit at the price of severe toxicities. Therefore, new agents and strategies are urgently needed to address Taxol-resistant breast cancer.

One approach to overcoming drug resistance is the use of drug copolymers. These high molecular weight conjugates can be transported via endocytosis to the endosome, where they are then cleaved to release free drug. For DNA-targeting drugs, this could afford superior nuclear access compared to import via diffusion as occurs with free drug. Further, it restricts the gradient of export of conjugate-released drug via membrane-localized drug efflux mechanisms that are clearly operant on free drug. In vivo, other considerations may be even more relevant, including distribution to tumor vs. normal tissue. High molecular weight drug copolymers may, on the one hand, 1) restrict diffusion-controlled uptake by normal tissues that occurs with free drug; but, on the other, they may 2) enhance extravasation across the abnormal tumor endothelium, thereby enhancing tumor localization compared to free drug. The paclitaxel copolymer employed in these studies, PGA-TXL, now commercially named "Xyotax", has shown both reduced toxicity and greater tumor localization in animal models, thereby fulfilling two expectations of copolymer behavior. To date, it has also demonstrated reduced toxicity and greater ease of administration compared to Taxol in the clinic, and has shown activity in patients with Taxol-refractory tumors. In this proposal, we will establish the toxicity, pharmacokinetics and anti-tumor efficacy of this Taxol copolymer in human breast adenocarcinoma models in nude mice; these models of P-gp- and HER-2/neu-mediated resistance will test the potency of the copolymer against resistance mechanisms that are operant against Taxol itself.

BODY

Task 1 Mechanistic Studies: Effects on Cell Cycle Distribution/Apoptosis and RAF-1 Kinase Activation

a) Conduct cell-cycle (PI staining) and apoptosis assays (TUNEL and hypodiploidy) on human breast adenocarcinoma cell lines (P-gp and HER-2/neu models) to be used in Task 4 to establish responses to Taxol and PGA-TXL in vivo

b) Using these cell lines and the doses established as relevant to previous endpoints, determine role of Raf-1 kinase pathway in these responses

These studies are currently underway with human HER-2/neu over-expressing MDA-MB-361 breast adenocarcinoma cells.

a) In initial studies with another apoptosis-inducing agent, dimethyl-sphingosine, we have observed that MDA-MB-361 cells rapidly, dose-dependently and progressively acquire PI-positivity, as well as positivity using the CaspaTag flow cytometric assay for pan-caspase activation. The CaspaTag-positive/PI-negative population increased by as much as ~30-fold compared to untreated controls, whereas the PI-positive population increased ~4-5-fold. Curiously, the increase in the CaspaTag-positive population appeared to initially lag behind that of the PI-positive population.

We anticipate similar patterns with regard to development of PI-staining and CaspaTag-staining following treatment with Taxol and PGA-TXL.

b) We have first undertaken these studies in a human ovarian carcinoma cell line, NMP-1, and are now conducting counterpart studies in MDA-MB-361 cells.

We have observed that the response to Taxol in NMP-1 cells is concentration- and time-dependent: 5 nM Taxol has minimal effects on cell cycle distribution at 24 hr, although it somewhat increases the hypodiploid population; this progresses to a robust G2/M block by 48 hr. At 25 nM, Taxol causes dramatic increases in the hypodiploid population as well as G2/M arrest, already by 24 hr.

The benzoquinone ansamycin, geldanamycin, which binds to the ATP binding pocket of HSP90, thereby inducing the degradation of proteins chaperoned by this HSP, has minimal effects on the cell cycle distribution of NMP-1 cells by itself, although it quenches S-phase cells at both 24 and 48 hr. When combined with 5 nM Taxol, it also appeared to quench the S-

phase population, without reducing the hypodiploid fraction induced by Taxol at 24 hr. When combined with 25 nM Taxol, geldanamycin markedly suppressed the development of hypodiploid cells incuded by this concentration of Taxol, indicating the requirement for HSP90-chaperoned proteins in the apoptotic response to Taxol. Studies by Torres and Horwitz (2) in a human lung carcinoma cell line have underscored a role for Raf-1 kinase in Taxol-induced apoptosis in this model; we will pursue this aspect in MDA-MB-361 cells should their response mirror this pattern.

We have also examined the CaspaTag response of NMP-1 cells to Taxol. At 4 hr, there was a ~50% increase in the CaspaTag-positive/PI-negative population in response to 5 nM Taxol, with minimal increases in PI-positive cells observed at this time; by 20-24 hr, there was a four-fold increase in the former population, and a ~60% increase in the latter (PI-positive) population. These results are consistent with induction of the caspase cascade preceding the development of hypodiploidy. Similar studies are underway in MDA-MB-361 cells.

Task 2 Pharmacokinetics: Cellular and IP Administration

a) Establish parameters of cellular uptake and fate of paclitaxel and PGA-TXL, using compounds ^{3}H-labeled in paclitaxel moiety or in PGA backbone; determine extent and site of PGA-TXL esterolysis to paclitaxel

b) Establish pharmacokinetic parameters for peritoneal clearance of paclitaxel and PGA-TXL following i.p. administration; determine parameters for resultant plasma levels compared to i.v. administration; determine extent and site of PGA-TXL esterolysis to paclitaxel

Studies related to this task have not yet been initiated.

Task 3 Toxicity Studies: Single- and Multiple-Dose IP and IV MTDs

a) Determine single-dose i.v. and i.p. MTD for PGA-TXL in nude mice

Task 4 Efficacy Studies: Her-2/neu- and P-gp-Mediated MDR Models

HER-2/neu-mediated MDR

a) Establish tumor responses and effects on survival of Taxol or PGA-TXL administered at single- or multiple-dose MTDs to nude mouse models of HER-2/neu high and basal expressing human breast adenocarcinomas

Initial activities relevant to these two Tasks were presented in the previous annual report; new studies pertinent to Task 4 are presented

below. The intent of the current study was to compare the anti-tumor efficacy of Taxol vs. PGA-TXL in the MDA-MB-361 model.

Poly(L-glutamic acid)-paclitaxel (PGA-TXL) was prepared by carbodiimide-mediated coupling of paclitaxel and poly(L-glutamic acid). Formulations of the final product contained ~20% paclitaxel (w/w), with a PGA backbone of ~30-40 k Da.

MDA-MB-361 human breast adenocarcinoma cells were obtained from the ATCC and were cultured exactly according to the ATCC-defined conditions and using their specific recommended serum. The cells were maintained in Liebowitz L-15 medium in the absence of CO_2. This allowed retention of original cellular morphology and growth pattern in vitro, albeit with a long doubling time (~7 days). Cells were finally trypsinized and adjusted to an inoculum cell number of 4-6 X 10^6 viable cells.

MDA-MB-361 cells were implanted under asceptic conditions in the mammary fat pad of 5-8 week old female nude mice. When tumor volumes reached a group average of ~20 mm^3 (Day 28 post-implantation; see below), one group of inoculated mice was treated with PGA-TXL. The formulation was injected i.p. in 100-200 microliters volume of PBS. A single dose level of 180 mg/kg was administered one time only. Two other groups received a multiple-dose regimen of either 5 or 10 mg/kg Taxol, near the MTD, and also administered i.p. Controls were given saline. Tumor outgrowth was evaluated by caliper measurement of perpendicular tumor diameters in treated and control groups. Animals with regressing tumors, in *either* control *or* treatment groups, were excluded from further evaluation; this severe censoring eliminated *any* concern that the spontaneous regressions occasionally observed in controls would be favorably and inadvertently factored into the responses to treatment. The group-averaged volume of these tumors, calculated as length x width x width/2, was normalized to the starting volume (at time of first treatment) and plotted vs. the day of measurement, through Day 63 post-implantation (35 days post-treatment initiation).

Control tumor volumes increased 5.89 +/- 0.43 -fold (mean +/- SEM) over this 35 day interval. Treatment with PGA-TXL was highly efficacious: the increase in tumor volumes in this group (11 mice) was only 2.96 +/- 0.31 -fold during the same time period ($p = 0.0006$ compared to controls; unpaired t-test). In contrast, the two Taxol-treated groups failed to demonstrate significant responses: tumor volumes increased 6.06 +/- 0.25 –fold ($p = 0.74$) and 4.29 +/- 0.61 -fold ($p = 0.101$) for the 5 and 10 mg/kg groups, respectively. However, their responses differed from each

other (p = 0.036) and the higher dose group nominally failed to be distinguishable (p = 0.056) from the PGA-TXL group. Therefore, and in the absence of evidence for toxicity with the 10 mg/kg dose level, in a future experiment to be conducted during the un-funded extension year, we will assess the response of the 361 model to a higher Taxol dose, likely 15 mg/kg.

KEY RESEARCH ACCOMPLISHMENTS

- The key results from this study indicate that even single-dose PGA-TXL is active against MDA-MB-361, an orthotopically-implanted human Her-2/neu over-expressing breast tumor model. Of note, this model appeared highly resistant to a multiple-dose regimen of Taxol.

REPORTABLE OUTCOMES

Three abstracts/presentations have arisen from this work since the commencement of the grant, and following verification and extension to a higher dose level of Taxol in the MDA-MB-361 model, we will prepare a manuscript.

1) The following abstract was submitted and accepted to the 6th US-Japan Symposium on Drug Delivery Systems, held in December, 2001 in Maui, HI. It was presented as a poster as well as being selected for presentation in a workshop.

PACLITAXEL COPOLYMER TO ADDRESS TAXOL RESISTANCE

J. Klostergaard, E. Auzenne, C. Li, N.J. Donato, M. Khodadadian, D. Farquhar, and Y. Zou

The University of Texas M.D. Anderson Cancer Center
Houston, Texas 77030 USA

We have evaluated a paclitaxel-poly(L-Glu) copolymer in human tumor/nude mouse orthotopic xenograft models which either reflect resistance to Taxol (HEY/ovarian) or over-express HER-2/neu (MDA-361/breast). Early treatment (Day 2 HEY) with MTD Taxol achieved some improvement in survival, but was not curative. However, treatment with copolymer markedly improved survival and some apparent cures were observed. The higher tumor burden at Day 7 rendered this model resistant to MTD Taxol, but still responsive to copolymer. Similarly, early treatment (Day 7) of the 361 breast model with paclitaxel copolymer resulted in substantial tumor growth delay, regression, or even apparent cure. When administered later, the copolymer still caused tumor growth delay, but no cures were observed. We conclude that

formulation of paclitaxel with this poly(L-Glu) backbone substantially enhanced its potency, and rendered it active in two highly drug-resistant models. Supported in part by DOD grants BC980420, BC991113 and OC000036 (JK).

2) The following abstract was presented as a poster at the Era of Hope Meeting, sponsored by the DOD Breast Cancer Research Program, in Orlando, FL, September 25th-28th, 2002.

LIPOSOMAL-DIMETHYL-SPHINGOSINE AND PACLITAXEL COPOLYMER ARE ACTIVE AGAINST HER-2/NEU OVER-EXPRESSING HUMAN BREAST ADENOCARCINOMA ORTHOTOPIC XENOGRAFT MODEL

Jim Klostergaard, Ph.D., Edmond Auzenne, M.S., C. Li, Ph.D., M. Khodadadian, B.S., David Farquhar, Ph.D. and Yiyu Zou, Ph.D.

The University of Texas
M.D. Anderson Cancer Center
Houston, TX 77339

E-mail: jkloster@mdanderson.org

Over-expression of HER-2/neu has been linked to poorer prognosis and survival in breast cancer patients. The basis for this association likely includes therapeutic resistance, including resistance to Taxol (paclitaxel), widely used in many chemotherapeutic regimens for this disease. We have recently observed that certain sphingolipids, either as free lipids or as constituents of liposomes, induce apoptosis in vitro in tumor cells despite the over-expression of Her-2/neu. Further, we have reported that a paclitaxel copolymer, paclitaxel-poly(L-glutamic acid) (PGA-TXL), is active against Taxol-resistant tumors in vivo.

We therefore evaluated liposomal-dimethyl-sphingosine (L-DMSP) and PGA-TXL in a human HER-2/neu over-expressing breast adenocarcinoma (MDA-361) orthotopic xenograft model. Tumor cells (4-6 X 10^6) were implanted in the mammary fat pad of 5-8 week old female nude mice. Mice were treated i.p. either one-week later or when tumors grew to 5-6 mm diameter.

Early treatment with a multiple-dose regimen of L-DMSP (4.5 mg DMSP per dose; 20 mole percent of a small unilamellar vesicle formulation), caused a delay in or reduced subsequent tumor growth, but was not curative. However, early treatment with a single-dose of PGA-TXL (180 mg/kg paclitaxel equivalents), also one week after tumor implantation, resulted in substantial tumor growth delay, regression, or even apparent cure in two of four mice (control tumor areas at 10 weeks post-implant = 44 ± 21.2 mm^2 ; treated group areas = 6 ± 6.0 mm^2). When administered at the later timepoint to another group of animals, PGA-TXL still caused tumor

growth delay, but no cures were observed (treated group areas = 24 ± 15.3 mm^2); nor did administration of L-DMSp at this time appear to be efficacious.

We conclude that DMSP as a liposomal formulation has some efficacy against this HER-2/neu over-expressing model when the tumor burden is low. Formulation of paclitaxel with the poly(L-glutamic acid) backbone substantially reduced its toxicity, enhanced its potency, and rendered it active against this HER-2/neu over-expressing breast adenocarcinoma model.

Supported by U.S. Army Medical Research and Material Command under DAMD17-99-1-9265 and DAMD17-00-1-0313.

3) The following abstract, submitted online, was accepted for presentation as a poster at the AACR/EORTC Meeting to be held in Frankfurt, Germany on November 19-23, 2002.

Therapeutic resistance to Taxol is a major issue in a number of cancers, particularly breast and ovarian carcinoma. This resistance is multifactorial, including P-gp170-linked MDR and over-expression of HER-2/neu. We evaluated the efficacy of a paclitaxel-poly(L-Glu) copolymer (PGA-TXL) in a human ovarian carcinoma orthotopic xenograft model which reflects resistance to Taxol (HEY); we also evaluated PGA-TXL as well as a liposomal (SUV) formulation of dimethyl-sphingosine (L-DMSP; which induces apoptosis in a broad spectrum of tumor cell lines *in vitro*) in an orthotopic human breast adenocarcinoma model that over-expresses HER-2/neu (MDA-361). In the ovarian model, early treatment (Day 2 post-implantation) with multiple-dose MTD Taxol (10 mg/kg) i.p. achieved slight improvement in survival, but was not curative. However, treatment with a single dose (180 mg/kg, paclitaxel equivalents) of PGA-TXL i.p. markedly improved survival and induced some apparent cures. The higher tumor burden present on Day 7 rendered this model resistant to MTD Taxol administration at this time, but still responsive to PGA-TXL. For the breast model, treatment on Day 7, before tumors were palpable, with PGA-TXL resulted in subsequent tumor growth delay, regression, or even apparent cure. Treatment at this time with a multiple-dose regimen of L-DMSP (4.5 mg DMSP/dose) i.p., caused a delay in or reduced subsequent tumor growth, but was not curative. When administered later after tumors grew to 5-6 mm diameter, PGA-TXL still caused tumor growth delay, but no cures were observed; administration of L-DMSP at this later time was not efficacious We conclude that formulation of paclitaxel with this poly(L-Glu) backbone substantially enhanced its potency, and rendered it active in drug-resistant ovarian and breast models. Further, we conclude that DMSP as a liposomal formulation has some efficacy against this HER-2/neu over-expressing breast model: however, only when the tumor burden is low. (Supported in part by DOD grants BC980420, BC991113 and OC000036 to JK).

CONCLUSIONS

HER-2/neu over-expression in breast cancer portends an aggressive clinical course and greater resistance to certain therapeutic regimens, including those involving taxanes. Although the advent of Herceptin has brought new opportunities for more effective and targeted therapy for women with this marker, other approaches must also be exploited. The use of a drug copolymer strategy for paclitaxel (Taxol) based on a poly(L-glutamic acid; PGA) backbone has proven in pre-clinical and clinical studies to reduce the toxicity of paclitaxel. Importantly, activity of PGA-TXL in Taxol-resistance settings has been observed, as well.

The key inference: our pre-clinical studies suggest that among the patients who could be considered for trials with PGA-TXL are those with tumors over-expressing HER-2/neu and refractory to conventional taxanes.

REFERENCES

1. Rowinsky EF and Donehower RC. Paclitaxel (Taxol) New Eng J Med 332; 1004-1014, 1995.
2. Torres K and Horwitz SB. Mechanisms of Taxol-induced cell death are concentration dependent. Cancer Res 58; 3620-3626, 1998.
3. Giannakakou P, Sackett DL, Kang Y-K, Zhirong Z, Buters JTM, Fojo T and Poruchynsky S. Paclitaxel resistant human ovarian cancer cells have mutant ß-tubulins that exhibit impaired paclitaxel-driven polymerization. J Biol Chem 272; 1711817125, 1997.
4. Yu D, Liu B, Tan M, Li J, Wang SS Hung M-C. Overexpression of c-erbB2/neu in breast cancer cells confers increased resistance to Taxol via mdr-1-independent mechanisms. Oncogene 13; 1359-65, 1996.
5. Yu D, Liu B, Jing T, Sun D, Price JE, Singletary SE, Ibrahim N, Hortobagyi GN and Hung M-C. Overexpression of both p185c-erbB2 and p170mdr-1 renders breast cancer cells highly resistant to taxol. Oncogene 16; 2087-2094, 1998.
6. Rowinsky EK,. On pushing the outer edge of the outer edge of paclitaxel's dosing envelope. Clin Cancer Res 5; 481-486, 1999.
7. Nabholtz J-M, Nabholtz J-M, Gelmon K, Bontenbal M, Spielmann M, Catiemel G, Conte P, Klaassen U, Namer M, Bonneterre J, Fumoleau P and Winograd B. Multicenter, randomized comparative study of two doses of paclitaxel in patients with metastatic breast cancer. J Clin Oncol 14; 1858-1867, 1996.

8. Winer E, Berry D, Duggan D, Henderson CI, Cirrincione C, Cooper R and Norton L. Failure of higher dose paclitaxel to improve outcome in patients with metastatic breast cancer: results from CALGB 9342. Proc Am Soc Clin Oncol 17; 101a, 1997.
9. Sparreboom A, van Tellingen O, Nooijen WJ and Beijnen JH. Nonlinear pharmacokinetics of paclitaxel in mice results from the pharmaceutical vehicle Cremophor EL. Cancer Res 56; 2112-2115, 1996.
10. Li C, Yu D-F, Newman RA, Cabral F, Stephens C, Hunter N, Milas L and Wallace S. Complete regression of well-established tumors using a novel water-soluble poly (L-glutamic acid)-paclitaxel conjugate. Cancer Res 58; 2404-2409, 1998.
11. Nicoletti MI, Lucchini V, Massazza G, Abbott BJ, D'Incalci M & Giavazzi R. Antitumor activity of taxol (NSC-125973) in human ovarian carcinomas growing in the peritoneal cavity of nude mice. *Ann Oncol* 4: 151-155, 1993.
12. Eiseman JL, eddington ND, Leslie L, McAuley C, Sentz DL, Zuhowsky M, Kujiwa JM, Young D and Egorin MJ. Plasma pharmacokinetics and tissues distribution of paclitaxel in CD2F1 mice. Cancer Chemother Pharmacol 34; 465-471, 1994.
13. McCloskey DE, Kaufmann SH, Prestigiacomo LJ and Davidson NE. Paclitaxel induces programmed cell death in MDA-MB-468 human breast cancer cells. Clin Cancer Res 2; 847-854, 1996.
14. Markman M, Rowinsky E, Hakes T, Reichman B, Jones W, Lewis JL, Jr, Rubin S, curtin J, Barakat R, Hurowitz L, Almadrones L and Hoskins W. Phase I trial of intraperitoneal Taxol: a gynecologic oncology group study. J Clin Oncol 10; 1485, 1992.

Resending

sent 11-11-04
via Fed Ex
8492-9819 6132

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO ATTENTION OF:

February 28, 2003

Research Data Management

SUBJECT: Review of Annual Report Dated July 2002,
Award Number DAMD17-00-1-0313

Jim Klostergaard, Ph.D.
Department of Molecular and Cellular Oncology, Box 108
The University of Texas M.D. Anderson Cancer Center
1515 Holcombe Boulevard
Houston, Texas 77030-4009

Dear Doctor Klostergaard:

Subject report has been reviewed and is acceptable as written. A copy of your report has been forwarded to the Defense Technical Information Center's Technical Reports database.

To assist you in preparing future reports under the subject award, we have enclosed the reviewer's comments. We ask that you review these comments and incorporate any recommended changes into future reports.

Point of contact for this action is Ms. Judy Pawlus at 301-619-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

Sincerely,

Judy Pawlus
Technical Editor

Enclosure

Submitted to Clinical Cancer Research

PACLITAXEL COPOLYMER ACTIVE AGAINST HIGHLY TAXOL-RESISTANT HER-2/NEU OVER-EXPRESSING HUMAN BREAST ADENOCARCINOMA ORTHOTOPIC XENOGRAFT MODEL

Edmond Auzenne[1], M. Khodadadian[1], Sukhen Ghosh[2], David Farquhar[2], Yiyu Zou[3] and Jim Klostergaard[1,4]

Departments of Molecular and Cellular Oncology[1] (Unit 108) and Experimental Therapeutics[2] (Unit 353)
The University of Texas, MD Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030 USA

Department of Oncology[3], Albert Einstein College of Medicine, Bronx, NY, 10461 USA

To whom correspondence should be addressed[4]

E-mail: jkloster@mdanderson.org

ABSTRACT

Over-expression of HER-2/*neu* has been linked to poorer prognosis and reduced survival in breast cancer patients. The basis for this association, although possibly multi-factorial, likely includes therapeutic resistance. We evaluated a novel paclitaxel copolymer, PGA-TXL, in a human HER-2/*neu* over-expressing breast adenocarcinoma (MDA-MB-361) orthotopic xenograft model. Both Taxol and PGA-TXL were cytotoxic to MDA-MB-361 cells *in vitro*, with Taxol being more potent. These cells responded to Taxol treatment by rapidly activating caspases and becoming permeable to propidium iodide; however, Taxol treatment did not result in HER-2/*neu* down-regulation. MDA-MB-361 cells were implanted in the mammary fat pad of 5-8 week old female nude mice; mice were treated intraperitoneally either one-week later or when tumors grew to 4-6 mm diameter. Treatment with a single-dose of PGA-TXL (180 mg/kg paclitaxel equivalents) one week after tumor implantation resulted in substantial tumor growth delay, regression or even apparent cure. Administration at the later time point also caused significant tumor growth delay, but no cures were observed. Under these treatment conditions for established tumors, the MDA-361 model was highly resistant to a multiple-dose regimen of Taxol, at up to 10 mg/kg, qd7 x 3. We concluded that formulation of paclitaxel with the PGA backbone substantially reduced the toxicity and enhanced the potency of paclitaxel, and rendered it more active than Taxol against this HER-2/neu over-expressing breast adenocarcinoma model.

INTRODUCTION

Most breast cancer treatment strategies depend on surgical intervention for debulking primary lesions, eradicating established nodal and, when possible, metastatic disease, in combination with radiation and chemotherapy. Among the available chemotherapeutic agents, Taxol, the clinical formulation of paclitaxel, has emerged as a clinically valuable chemotherapeutic agent, used either alone or in drug combinations. Improved survival and disease-free intervals have been achieved with Taxol regimens.

Nevertheless, multifactorial mechanisms of Taxol-resistance have been demonstrated in numerous laboratory and clinical studies, including over-expression of HER-2/*neu* (1-4). Drug resistance may in part be linked to constitutive HER-2/neu-mediated activation of Akt-induced anti-apoptotic/pro-survival mechanisms (5-11). Several recent clinical trials have suggested that dose-intensification with Taxol to overcome resistance may be a situation of diminishing returns (12-14). In two randomized breast cancer clinical trials, escalating doses of Taxol from 135 to 175 mg/m^2 (13) or 175 to 250 mg/m^2 (14) using 3 hr infusion schedules failed to demonstrate marked improvement in disease response or survival, despite incurring severe sensory neurotoxicity and myelosuppression. Therefore, it is important to develop new agents and approaches that might address Taxol-resistance.

One approach under development for overcoming drug resistance involves macromolecular polymeric conjugates of chemotherapeutic agents. Macromolecular drugs are internalized by endocytosis, which results in their accumulation in the perinuclear lysosomes. Such drug copolymers may be more advantageous than free drugs, since free drugs readily extravasate to normal tissues, whereas drug copolymers do not because of their large size. However, these copolymers can still traverse the leaky, irregular vasculature of solid tumors, allowing superior

tumor localization and less toxicity than with free drugs. These results are predictable because of the so-called "enhanced permeability and retention (EPR) mechanism" (15-18).

Paclitaxel has been formulated as a water-soluble, copolymer conjugate using a poly-L-glutamic acid backbone (PGA-TXL; 19). Characterization of PGA-TXL has revealed remarkable *in vivo* properties in several tumor models, including reduced toxicity, greater tumor localization, and superior antitumor efficacy compared to Taxol, including cures in Taxol-resistant tumor models (19-23). Encouraged by such pre-clinical data, clinical trials were initiated a few years ago with the clinical formulation of PGA-TXL, XYOTAX™, including ongoing phase II and III trials.

In the MDA-MB-435Lung2 xenograft model derived from HER-2/*neu*-low-expressing human breast adenocarcinoma, Li and coworkers demonstrated the superior anti-tumor efficacy of PGA-TXL over Taxol (19). In a later *in vitro* study of the HER-2/*neu*-over-expressing MDA-MB-453) human breast adenocarcinoma model, Oldham et al. (24) observed that both PGA-TXL and Taxol down-regulated HER-2/*neu* expression. However, they found that contrary to the expected correlation of Taxol resistance with HER-2/*neu* over-expression, the HER-2/*neu*-low-expressing MCF-7 cells were more resistant to the drugs than were the HER-2/*neu*-overexpressing MDA-MB-453 cells. In the 453 cells, both drugs down-regulated HER-2/*neu* expression, and this down-regulation may have allowed apoptosis to proceed. However, in that study, the tumor was exposed continuously to high (1 μM) drug concentrations that were well above clinically relevant levels (25-29). Thus, the significance of this down-regulation to an apoptotic outcome remains unclear.

To date, no study has evaluated the efficacy of PGA-TXL in a preclinical model of HER-2/*neu*-overexpressing human breast adenocarcinoma. Therefore, in our study, we used a xenograft model of an established HER-2/*neu* over-expressing human breast adenocarcinoma,

MDA-MB-361, to compare the antitumor efficacy of PGA-TXL and Taxol. We also investigated whether HER-2/*neu* down-regulation by paclitaxel, a possible mechanism for overcoming HER-2/*neu*-mediated drug resistance, would occur when pharmacologically relevant drug doses were given.

MATERIALS & METHODS

Cell Line

The MDA-MB-361 cell line, a HER-2/*neu*-overexpressing human breast adenocarcinoma, was obtained from ATCC (Manassas, VA; catalogue # HTB 27) and was maintained according to their recommendations in Leibovitz's L-15 Medium supplemented with 10% fetal bovine serum (ATCC) under CO_2-free conditions. The doubling time under these conditions was 5-7 days. Any deviation from these conditions, particularly introduction of CO_2, resulted in altered cellular morphology or even loss of viability.

Cytotoxicity Assay

Subconfluent monolayers of MDA-MB-361 tumor cells were established in wells of 96-well plates. After overnight incubation, cells were exposed to a range of concentrations up to 500 ng/ml of Taxol (Bristol Myers Squibb) or PGA-TXL (paclitaxel equivalents). After further incubation for up to 120 hr, the cells were stained with MTT or neutral red. After solubilizing incorporated dye, the absorbance at 570 or 540 nm, respectively, was determined. Survival was calculated as the absorbance in wells of treated cells, normalized to controls.

Flow Assays

Caspase activity in the MDA-MB-361 cells was determined using the flow cytometry CaspaTag activity kit (Intergen, Purchase, NY), based on a fluorescein-labeled caspase inhibitor that is cell-permeable and covalently binds only active caspases. Briefly described: MDA-MB-361 cells were treated with a Taxol concentration of up to 25 nM for 24 hr. Then the cells were washed and resuspended in warmed, complete RPMI, supplemented with the fluorochrome-peptide-fmk CaspaTag reagent and propidium iodide for 1 h at 37°C. Cells were washed twice, resuspended in fixing buffer, and analyzed immediately by flow cytometry.

Immunoblotting

The anti-human HER-2/*neu* mouse monoclonal antibody, clone 3B5, isotype IgG1, was obtained from Oncogene Research Products (San Diego, CA). It was prepared by mouse immunization against a 14 amino acid peptide sequence (TAENPEYLGLDVPV) in the carboxyl domain of the human c-*neu* gene product.

An affinity-purified sheep anti-mouse IgG whole antibody preparation conjugated to horseradish peroxidase for ECL was obtained from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ). Broad range (6.5-175 kDa) prestained protein markers for use in gels and biotinylated protein detections markers (6.5-165 kDa) for Western blotting were from Cell Signaling Technology.

Lysates of MDA-MB-361 cells (1.5-3.0 X 10^6 cells plated in a 10 cm dish on Day 0), which were cultured without treatment (controls) or following treatment with Taxol (30-300 nM) for up to 48 hr, were prepared by first collecting detached cells by decanting, washing the tissue culture plates with PBS, lysing the attached cells with lysis buffer (20% SDS, 0.5 M Tris Phosphate, 10% glycerol, 2 ul/ml PMSF, 3 ul/ml Protease cocktail (0.67 M EDTA, 333 uM pepstatin, 333 uM leupeptin). and centrifugation of all combined lysates. Protein assay was performed by Bradford microassay. Equal protein loads (25 or 50 ug) were placed in wells of gels prior to electrophoresis and subsequent transfer and immunoblotting using Advanced ECL technique kit (Amersham Biosciences, Cat# RPN 2135), with primary anti-HER-2/neu antibody (C-Neu, cat # OP15-100uG, Oncogene) at a 1:5000 dilution and a secondary antibody (sheep anti-mouse Ig linked to horseradish peroxidase (Amersham Biosicences).

PGA-TXL Synthesis

PGA-TXL was prepared by carbodiimide-mediated coupling of paclitaxel and poly(L-glutamic acid), according to previous techniques (22, 23). Formulations of the final product contained ~20% paclitaxel (w/w), with a PGA backbone of ~30-40 k Da.

Orthotopic Xenograft model

MDA-361 human breast adenocarcinoma cells (4-6 X 10^6) were implanted in the mammary fat pad of 5-8 week old female nude mice. Mice were treated intraperitoneally with either a multiple-dose regimen of Taxol (5 or 10 mg/kg), qd7 X 3, or a single dose of PGA-TXL (180 mg/kg, paclitaxel equivalents) beginning either one-week after tumor implantation or when tumors grew to 4-6 mm diameter. The tumor diameters were measured by caliper until either a tumor diameter reached 1.5 cm, or the host presented with moribund symptoms, and required host sacrifice. The diameter measurements were used to calculate area (l x w) or volume (l x w x w ÷ 2). Raw diameter or volume values were used directly or were normalized to the starting values for each group.

Statistics

Selected statistical analyses of the data were done by analysis of variance (ANOVA, Statview 4:01, Abacus Concepts, Inc., Berkeley, CA), and post-hoc testing by Fisher's protected least significant difference (Fisher's PLSD, Statview 4.01) at p=0.05. The reported data was based on entering 4 to 12 mice per treatment group.

RESULTS

Sensitivity of MDA-MB-361 cells to Taxol and PGA-TXL *in vitro*

To establish the *in vitro* sensitivity of the MDA-MB-361 cell line to paclitaxel and PGA-TXL, tumor cells were exposed to up to 500 ng/ml (586 nM) of Taxol or PGA-TXL (paclitaxel equivalents). After incubation for 120 hr of continuous drug exposure, cell survival was determined and is shown in **Figure 1**. Taxol achieved an IC_{50} at ~ 35 nM at this time point, whereas even the highest concentration of PGA-TXL (586 nM) did not achieve an IC_{50}; the response to PGA-TXL reached a plateau of ~60-70% survival with a concentration of 125-500 ng/ml (147-586 nM).

This behavior suggested a reduced or more protracted response of MDA-MB-361 cells to the paclitaxel prodrug than with free paclitaxel itself, as might be expected in light of the internalization and activation steps for the former; further, this effect of PGA-TXL is consistent with minimal extracellular cleavage of the prodrug to free paclitaxel, in contrast to previous studies (24).

Flow Assays for Caspase Activation and Cell Death of MDA-MB-361 cells by Taxol Treatment *in vitro*

Caspase activity induced in the MDA-MB-361 cell line in response to 24 hr treatment with up to 25 nM Taxol was determined by the CaspaTag assay and the results are shown in **Table 1**. Taxol induced concentration-dependent increases in both Caspase(+) and PI(+) cells. The highest concentration (25 nM) caused an approximately two-fold increase in Caspase(+) cells, both those that were still PI(-) (5.4% vs. 2.8%) and in total Caspase(+) cells (7.1% vs. 3.6%). The increase in the total number of PI(+) cells with this Taxol concentration and at this time point was about five-fold (13.7% vs. 2.8%).

This result suggested that the mechanism of Taxol-mediated cell death paralleled early caspase activation in these cells.

Regulation of HER-2/neu Expression in MDA-MB-361 cells by Paclitaxel *in vitro*

Previous studies with the HER-2/*neu* over-expressing human breast adenocarcinoma cell line, MDA-MB-453 (24), indicated that both paclitaxel and PGA-TXL treatments down-regulated HER-2/neu expression, as a possible mechanism for overcoming Her-2/*neu*-mediated resistance. We determined whether the MDA-MB-361 cell line responded in a similar fashion.

Lysates of MDA-MB-361 cells that had been cultured for 24 or 48 hr without treatment (controls) or with Taxol (30-300 nM), the low range of which had been previously shown to induce signifiant cell death even in 24 hr (**Table 1**), were prepared. The results of immunoblotting with anti-HER-2/*neu* antibody following electrophoresis and subsequent transfer are shown in **Figure 2.**

There was *no* detectable decrease in the intensity of the HER-2/*neu* expression in cells that had been treated with as high as 300 nM paclitaxel for 24 hr (lanes 1-3) or 48 hr (lanes 5-7), compared to untreated controls (lane 4). Thus, paclitaxel-induced decreases in HER-2/*neu* expression did not precede cell death *in vitro* in this model.

Anti-Tumor Activity of Taxol and PGA-TXL Against MDA-MB-361 Orthotopic Xenograft Model

MDA-MB-361 cells were implanted orthotopically in nude mice. In a pilot study to determine the efficacy of PGA-TXL against nascent versus established tumors, early treatment with a single-dose of PGA-TXL (180 mg/kg paclitaxel equivalents) one week after tumor implantation caused substantial tumor growth inhibition and two of four mice were apparently cured (**Figure 3**). At the termination of the experiment on Day 107 post-implantation, the difference between tumor areas of control and treated mice was significant ($p = 0.021$). The

tumor growth curve following this single PGA-TXL treatment was primarily suggestive of stasis, with anti-tumor effects evident for as long as ~40 days after this treatment. Only slight further growth occurred in the next 30 days, reflecting a much slower growth rate than for controls.

When PGA-TXL was instead administered at the later timepoint (average tumor diameters, ~4-5 mm), treatment with PGA-TXL was still efficacious against the higher tumor burden, but less so than with early treatment. Partly due to the small sample size in this pilot study, the response failed to achieve significance ($p = 0.119$). The tumor growth curve following this single treatment was again suggestive of stasis at the initial tumor volume, suggesting a rapid onset of PGA-TXL-mediated effects, with these effects evident for as long as ~30 days after this single treatment (**Figure 3**). Further growth occurred in the next 40 days, more aggressively than observed when tumors were treated early, but still somewhat slower than for controls.

The absence of tumors observed in some of the mice in the early treatment group of this pilot study was intriguing. However, we have observed that the frequency of tumor outgrowth after orthotopic implantation of MDA-MB-361 cells is at most ~85-90%; thus, the lack of tumor outgrowth in these treated mice could have been anomalous and simply due to an expected outgrowth failure rate. Further, in this model, some tumors that had initially grown to 2-3 mm diameter by 5-6 weeks spontaneously regressed. Therefore, in more definitive studies focused on the treatment of established MDA-MB-361 tumors, two conditions were met; treatment was withheld until tumor diameters were at least 4-6 millimeters diameter, the point after which spontaneous regression is almost never observed; further, the group size of the PGA-TXL treatment arm was increased to 11 evaluated mice. The three treatment groups included Taxol, administered intraperitoneally at either 5 or 10 mg/kg, qd7 x 3, or PGA-TXL, administered in a single intraperitoneal injection at 180 mg/kg (paclitaxel equivalents). The tumor volume data normalized to starting values for this experiment are shown in **Figure 4**.

Control tumor volumes increased ~6.2-fold over this six week period of observation. The volumes for the lower dose (5 mg/kg) Taxol group increased ~6.5-fold in the same period ($p = 0.74$ vs. control; $p < 0.001$ vs. PGA-TXL), reflecting tumor resistance to this dose and schedule. The higher dose (10 mg/kg) Taxol group displayed a lower increase in tumor volume, ~3.9-fold ($p = 0.1$ vs. control; $p = 0.35$ vs. lower dose Taxol group), suggesting marginal activity. Of note, the tumor growth curve for this Taxol group only plateaued and departed from the control curve well after the third injection of this regimen, suggesting the benefit of the multiple-dose schedule. Finally, the PGA-TXL group displayed a volume increase of only ~3.0-fold by the end of this observation period ($p = 0.0006$ vs. control; $p = 0.06$ vs. high dose Taxol group). Possibly due to the somewhat larger tumors treated in this study compared to the late treatment group of the pilot study, no stasis at the initial tumor volume was observed: rather, only a slower growth rate than for the controls. Thus, in this model a single treatment with PGA-TXL was clearly more efficacious than a multiple-dose regimen of Taxol at either 5 or 10 mg/kg, although in contrast to the early treatment study (**Figure 3**), with no apparent cures, and with every individual mouse demonstrating progression.

DISCUSSION

The results in this study in the MDA-MB-361 HER-2/*neu* over-expressing human breast adenocarcinoma xenograft model demonstrate the marked anti-tumor efficacy of administration of even a single injection of PGA-TXL, whereas multiple-dose, near-MTD regimens of Taxol had either no or at best marginal efficacy in this model. We conclude that the formulation of paclitaxel with the PGA backbone substantially reduced the toxicity and enhanced the potency of paclitaxel, and rendered it more active than Taxol against this HER-2/*neu*-overexpressing breast adenocarcinoma model. These results provide the first preclinical evidence that PGA-TXL should possibly be considered for clinical evaluation in the subpopulation of breast cancer patients that over-express HER-2/*neu*.

Previous pre-clinical studies have evaluated PGA-TXL in the orthotopic MDA-MB-435Lung2 xenograft model (20). This study demonstrated that PGA-TXL had superior anti-tumor activity compared to Taxol against both the primary tumor and against pulmonary metastases, which are important observations. However, although the MDA-MB-435 model is of interest as it reflects the metastatic nature of human breast cancer, it is a low expresser of HER-2/*neu*, so that HER-2/*neu*-mediated resistance to paclitaxel would not be evident in this particular study.

In the current studies, we did not evaluate the anti-tumor efficacy of multiple dose regimens of PGA-TXL, nor did we conduct any toxicology experiments. With regard to the former, curiously, previous studies using multiple-dose regimens of PGA-TXL with either loco-regional or systemic administration did not demonstrate marked improvement in tumor response compared to single treatment (20, 22); the basis for this counter-intuitive result is uncertain, but further exploration of optimal scheduling may yet result in therapeutic gain. With regard to toxicology, in light of the advanced state of clinical evaluation of the clinical formulation of

PGA-TXL (see below), we believed that additional pre-clinical toxicological evaluation in the context of our current studies would be of minimal significance. These clinical studies have revealed a similar profile of normal tissue toxicities for PGA-TXL compared to Taxol, such as allergic responses, neuropathies and transient neutropenia, albeit with a single-agent MTD ≥ 30% higher than for Taxol.

The Oldham study evaluated the activity of paclitaxel and PGA-TXL against HER/2neu over-expressing MDA-MB-453 cells *in vitro* (24). These investigators observed that these agents were very similar to each other in their ability to induce G2/M arrest and apoptosis in MCF-7, MDA-MB-435 or MDA-MB-453 cells. Further, both agents caused down-regulation of HER-2/*neu* protein expression in HER-2/*neu* over-expressing MDA-MB-453 cells, in contrast to our current results with HER-2/*neu* over-expressing MDA-MB-361 cells (**Figure 2**). However, these investigators used these drugs at a concentration of 1 uM for up to 48 hr of continuous treatment, conditions unlikely to be achieved *in vivo* (25-29); so the real significance of this *in vitro* down-regulation is uncertain. Further, both pre-clinical and clinical evidence indicates that HER-2/*neu* over-expression portends a *superior* response to taxane-based therapy than observed with patients without HER-2/*neu* amplification (30-32). Interestingly, this clinical data appears to be in conflict with other studies that have linked HER-2/*neu* over-expression to tumor cell resistance to paclitaxel (1-4). Alternatively, this discrepancy may suggest that therapeutic outcome depends on factors other than solely the tumor cell sensitivity to paclitaxel, such as paclitaxel-mediated effects on tumor vasculature (33-36). Indeed, Taxol and particularly certain liposomal formulations of paclitaxel have been reported to target tumor vasculature (35).

Since the clinical introduction of Tratuzumab/Herceptin, efforts have been made to prospectively rationalize and evaluate the combination of this monoclonal antibody with conventional chemotherapeutic agents, including Taxol (37-44). In light of the demonstrated

clinical efficacy of Herceptin/Taxol combination regimens, we propose that PGA-TXL should also be considered for evaluation for anti-tumor efficacy in combination with Herceptin, initially in pre-clinical HER-2/*neu* over-expressing breast tumor models.

XYOTAX™, the clinical formulation of PGA-TXL, is currently in advanced phase II and phase III clinical evaluations in lung cancer and ovarian cancer trials (45-47). In summary, our pre-clinical data suggest that evaluation of XYOTAX™ in the HER-2/*neu* over-expressing subpopulation of breast cancer patients may also be warranted, particularly in light of the highly aggressive and resistant disease associated with this over-expression.

ACKNOWLEDGMENTS

These studies were supported by the Department of Defense Breast Cancer Research Program, Award DAMD17-00-1-0313. We thank Gayle Nesom in Scientific Publications for valuable editorial assistance.

REFERENCES

1. Yu D, Liu B, Tan M, Li J, Wang SS Hung M-C. Overexpression of c-erbB2/neu in breast cancer cells confers increased resistance to Taxol via mdr-1-independent mechanisms. Oncogene 13; 1359-65, 1996.

2. Yu D, Liu B, Jing T, Sun D, Price JE, Singletary SE, Ibrahim N, Hortobagyi GN and Hung M-C. Overexpression of both p185c-erbB2 and p170mdr-1 renders breast cancer cells highly resistant to taxol. Oncogene 16; 2087-2094, 1998.

3. Tan M, Jing T, Lan KH, Neal CL, Li P, Lee S, Fang D, Nagata Y, Liu J, Arlinghaus R, Hung MC, Yu D. Phosphorylation on tyrosine-15 of p34(Cdc2) by ErbB2 inhibits p34(Cdc2) activation and is involved in resistance to taxol-induced apoptosis. *Mol Cell.* 5: 993-1004, 2002.

4. Witters LM, Santala SM, Engle L, Chinchilli V, Lipton A. Decreased response to paclitaxel versus docetaxel in HER-2/neu transfected human breast cancer cells. *Am J Clin Oncol.* 26: 50-54, 2003.

5. Zhou BP, Hu MC, Miller SA, Yu Z, Xia W, Lin SY, Hung MC. HER-2/neu blocks tumor necrosis factor-induced apoptosis via the Akt/NF-kappaB pathway. *J Biol Chem.* 275: 8027-8031, 2000.

6. Bacus SS, Altomare DA, Lyass L, Chin DM, Farrell MP, Gurova K, Gudkov A, Testa JR. AKT2 is frequently upregulated in HER-2/neu-positive breast cancers and may contribute to tumor aggressiveness by enhancing cell survival. *Oncogene.* 21: 3532-3540, 2002.

7. Siwak DR, Mendoza-Gamboa E, Tari AM. HER2/neu uses Akt to suppress retinoic acid response element binding activity in MDA-MB-453 breast cancer cells. *Int J Oncol.* 23: 1739-1745, 2003.

8. Klos KS, Zhou X, Lee S, Zhang L, Yang W, Nagata Y, Yu D. Combined trastuzumab and paclitaxel treatment better inhibits ErbB-2-mediated angiogenesis in breast carcinoma through a more effective inhibition of Akt than either treatment alone. *Cancer.* 98: 1377-1385, 2003.

9. Holbro T, Beerli RR, Maurer F, Koziczak M, Barbas CF 3rd, Hynes NE. The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation. *Proc Natl Acad Sci U S A.* 100: 8933-8938, 2003.

10. Knuefermann C, Lu Y, Liu B, Jin W, Liang K, Wu L, Schmidt M, Mills GB, Mendelsohn J, Fan Z. HER2/PI-3K/Akt activation leads to a multidrug resistance in human breast adenocarcinoma cells. *Oncogene.* 22: 3205-3212, 2003.

11. Stoica GE, Franke TF, Wellstein A, Czubayko F, List HJ, Reiter R, Morgan E, Martin MB, Stoica A. Estradiol rapidly activates Akt via the ErbB2 signaling pathway. *Mol Endocrinol.* 17: 818-830, 2003

12. Rowinsky EK. On pushing the outer edge of the outer edge of paclitaxel's dosing envelope. *Clin Cancer Res* 5; 481-486, 1999.

13. Nabholtz J-M, Nabholtz J-M, Gelmon K, Bontenbal M, Spielmann M, Catiemel G, Conte P, Klaassen U, Namer M, Bonneterre J, Fumoleau P and Winograd B. Multicenter, randomized comparative study of two doses of paclitaxel in patients with metastatic breast cancer. *J Clin Oncol 14*; 1858-1867, 1996.

14. Winer E, Berry D, Duggan D, Henderson CI, Cirrincione C, Cooper R and Norton L. Failure of higher dose paclitaxel to improve outcome in patients with metastatic breast cancer: results from CALGB 9342. *Proc Am Soc Clin Oncol* 17; 101a, 1997.

15. Omelyanenko V, Kopeckova P, Gentry C and Kopecek J. Targetable HPMA copolymer-adriamycin conjugates. Recognition, internalization, and subcellular fate. *J Control Release* 53: 25-37, 1998.

16. Seymour L W. Passive tumor targeting of soluble macromolecules and drug conjugates. *CRC Crit Rev Ther Drug Carrier Syst* 9: 135-187, 1992.

17. Brown J M and Giaccia A J. The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. *Cancer Res* 58: 1408-1416, 1998.

18. Greish K, Fang J, Inutsuka T, Nagamitsu A, Maeda H. Macromolecular therapeutics: advantages and prospects with special emphasis on solid tumor targeting. *Clin Pharmacokinet.* : 1089-105, 2003.

19. Li C, Yu D-F, Newman RA, Cabral F, Stephens C, Hunter N, Milas L and Wallace S. Complete regression of well-established tumors using a novel water-soluble poly (L-glutamic acid)-paclitaxel conjugate. Cancer Res 58; 2404-2409, 1998.

20. Li C., Price J. E., Milas L., Hunter N. R., Ke S., Yu D.-F., Charnsangavej C. and Wallace S. Antitumor activity of poly(L-glutamic acid)-paclitaxel on syngeneic and xenografted tumors. *Clin. Cancer Res.* 5: 891-897, 1999.

21. Li C., Ke S., Wu Q.-P., Tansey W., Hunter N., Buchmiller L. M., Milas L., Charnsangavej C. and Wallace S. Tumor irradiation enhances the tumor-specific distribution of poly(L-glutamic acid)-conjugated paclitaxel and its antitumor efficacy. *Clin Cancer Res* 6: 2829-2834, 2000.

22. Auzenne, E., Donato, N.J.,Li, C., Price, R., Leroux, E., Farquhar D. and Klostergaard, J. Superior therapeutic profile of poly-L-glutamic acid-paclitaxel copolymer compared to Taxol in xenogeneic compartmental models of human ovarian carcinoma. *Clin Cancer Res.* 8: 573-581, 2002.

23. Zou Y, Fu H, Ghosh S, Farquhar[D] and Klostergaard J. Antitumor Activity of Hydrophilic Paclitaxel Copolymer Prodrug Using Locoregional Delivery in Human Orthotopic NSCLC Xenograft Models. *Clin Cancer Res.* (in press).

24. Oldham EA, Li C, Ke S, Wallace S, Huang P. Comparison of action of paclitaxel and poly(L-glutamic acid)-paclitaxel conjugate in human breast cancer cells. *Int J Oncol.* 16: 125-132, 2000.

25. Mross K, Haring B, Hollander N, Mielke S, Behringer D, Massing U, Unger C. Comparison of 1-hour and 3-hours paclitaxel infusion pharmacokinetics: results from a randomized trial. *Onkologie*. 25: 503-508, 2002.

26. Leyland-Jones B, Gelmon K, Ayoub JP, Arnold A, Verma S, Dias R, Ghahramani P. Pharmacokinetics, safety, and efficacy of trastuzumab administered every three weeks in combination with paclitaxel. *J Clin Oncol.* 21: 3965-3671, 2003.

27. Campos SM, Matulonis UA, Penson RT, Lee H, Berkowitz RS, Duska LR, Fuller AF Jr, Wilson KS, Puchalski TA, Supko JG, Seiden MV. Phase II study of liposomal doxorubicin and weekly paclitaxel for recurrent Mullerian tumors. *Gynecol Oncol.* 90: 610-618, 2003.

28. Henningsson A, Sparreboom A, Sandstrom M, Freijs A, Larsson R, Bergh J, Nygren P, Karlsson MO. Population pharmacokinetic modelling of unbound and total plasma concentrations of paclitaxel in cancer patients. *Eur J Cancer.* 39: 1105-1114, 2003.

29. Urabe S, Fujiwara H, Egawa M, Fujitou N, Sakashita T, Date K, Mizunoe T, Ueda K, Urabe T. Recurrent ovarian cancer with cancer peritonitis treated with weekly paclitaxel infusion--a clinicopharmacological study. *Gan To Kagaku Ryoho.* 31: 449-451, 2004.

30. Gianni L Future directions of paclitaxel-based therapy of breast cancer. *Semin Oncol.* 5 Suppl 17:S17-91-S17-96, 1997.

31. Baselga J, Seidman AD, Rosen PP, Norton L. HER2 over-expression and paclitaxel sensitivity in breast cancer: therapeutic implications. *Oncology (Huntingt).* 3 Suppl 2: 43-48, 1997.

32. Konecny GE, Thomssen C, Luck HJ, Untch M, Wang HJ, Kuhn W, Eidtmann H, du Bois A, Olbricht S, Steinfeld D, Mobus V, von Minckwitz G, Dandekar S, Ramos L, Pauletti G, Pegram MD, Janicke F, Slamon DJ. Her-2/neu gene amplification and response to paclitaxel in patients with metastatic breast cancer. *J Natl Cancer Inst.* 96: 1141-1151, 2004.

33. Lau DH, Xue L, Young LJ, Burke PA, Cheung AT. Paclitaxel (Taxol): an inhibitor of angiogenesis in a highly vascularized transgenic breast cancer. *Cancer Biother Radiopharm.* 14: 31-36, 1999.

34. Hayot C, Farinelle S, De Decker R, Decaestecker C, Darro F, Kiss R, Van Damme M. In vitro pharmacological characterizations of the anti-angiogenic and anti-tumor cell migration properties mediated by microtubule-affecting drugs, with special emphasis on the organization of the actin cytoskeleton. *Int J Oncol.* 21: 417-425, 2002.

35. Schmitt-Sody M, Strieth S, Krasnici S, Sauer B, Schulze B, Teifel M, Michaelis U, Naujoks K, Dellian M. Neovascular targeting therapy: paclitaxel encapsulated in cationic liposomes improves antitumoral efficacy. *Clin Cancer Res.* 9: 2335-41, 2003.

36. Drevs J, Fakler J, Eisele S, Medinger M, Bing G, Esser N, Marme D, Unger C. Antiangiogenic potency of various chemotherapeutic drugs for metronomic chemotherapy. *Anticancer Res.* 24: 1759-1763, 2004.

37. Baselga J, Norton L, Albanell J, Kim Y-M and Mendelsohn J. Recombinant humanized anti-HER2 antibody (Herecptin™) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/*neu* overexpressing human breast cancer xenografts. Cancer Res 58; 2825-2831, 1998.

38. Dieras V, Beuzeboc P, Laurence V, Pierga JY, Pouillart P. Interaction between Herceptin and taxanes. *Oncology.* 61 Suppl 2: 43-49, 2001.

39. Nabholtz JM, Reese DM, Lindsay MA, Riva A. HER2-positive breast cancer: update on Breast Cancer International Research Group trials. *Clin Breast Cancer.* 3 Suppl 2: S75-79, 2002.

40. Ligibel JA and Winer EP. Trastuzumab/chemotherapy combinations in metastatic breast cancer. *Semin Oncol.* 3 Suppl 11: 38-43, 2002.

41. Campone M, Merlin JL, Fumoleau P. Role of the combination of trastuzumab and taxanes in the therapeutic management of cancer of the breast: from preclinical data to clinical application. *Bull Cancer*. 90: 614-622, 2003.

42. Christodoulou C, Klouvas G, Pateli A, Mellou S, Sgouros J, Skarlos DV. Prolonged administration of weekly paclitaxel and trastuzumab in patients with advanced breast cancer. *Anticancer Res.* 23 (1B): 737-744, 2003.

43. Burstein HJ, Harris LN, Gelman R, Lester SC, Nunes RA, Kaelin CM, Parker LM, Ellisen LW, Kuter I, Gadd MA, Christian RL, Kennedy PR, Borges VF, Bunnell CA, Younger J, Smith BL, Winer EP. Preoperative therapy with trastuzumab and paclitaxel followed by sequential adjuvant doxorubicin/cyclophosphamide for HER2 overexpressing stage II or III breast cancer: a pilot study. *J Clin Oncol.* 21: 46-53, 2003.

44. Pegram MD, Konecny GE, O'Callaghan C, Beryt M, Pietras R, Slamon DJ. Rational combinations of trastuzumab with chemotherapeutic drugs used in the treatment of breast cancer. *J Natl Cancer Inst.* 96: 739-749, 2004.

45. Garner P. (ed.) Phase II clinical trial of XYOTAX™ in non-small cell lung cancer to continue. *Expert Rev Anticancer Ther.* 2: 244-245, 2002.

46. CT-2103 extends median survival of high-risk lung cancer patients. *Oncology (Huntingt).* 17: 1804, 1809, 2003.

47. http://www.celltherapeutics.com

FIGURE LEGENDS

Figure 1 Dose-response of MDA-MB-361 cells to Taxol and PGA-TXL

MDA-MB-361 cells were exposed to up to 500 ng/ml of Taxol or PGA-TXL (paclitaxel equivalents). After 120 hr, cell survival was determined and normalized to controls. Taxol achieved an IC_{50} of ~35 nM; in contrast, PGA-TXL did not achieve an IC_{50}, even at the highest concentrations.

Figure 2 HER-2/neu expression of MDA-MB-361 cells after Taxol treatment

Lysates of MDA-MB-361 cells previously cultured without treatment (controls) or after treatment with Taxol (30, 100, and 300 nM) for 24 or 48 hr were subjected to electrophoresis and subsequent transfer and immunoblotting using an anti-HER-2/neu antibody. No alteration was evident in the intensity of the HER-2/*neu* expression in the cells that had been treated with 300, 100, or 30 nM paclitaxel for 24 hr (lanes 1, 2, and 3, respectively) or 48 hr (lanes 5, 6, and 7, respectively), compared with that in the controls (lane 4).

Figure 3 Responses of MDA-MB-361 model to early or late PGA-TXL

MDA-MB-361 cells were implanted in the mammary fat pads of female nude mice that were subsequently treated with a single dose of PGA-TXL (i.p.; 180 mg/kg, paclitaxel equivalents) beginning either one week after tumor implantation (early) or when the tumors grew to 4-5 mm in diameter (late). The tumor diameters were measured by caliper and were used to calculate area (l x w). With the early treatment, substantial tumor growth inhibition was observed, including even apparent cure in two of four mice. At Day 107 post-implantation, the difference between the tumor areas of the controls and the treated mice was significant ($p=0.021$). With the late

treatment, PGA-TXL was still slightly efficacious against the higher tumor burden, but the response failed to achieve significance (p=0.119).

Figure 4 Responses of MDA-MB-361 model to Taxol and PGA-TXL

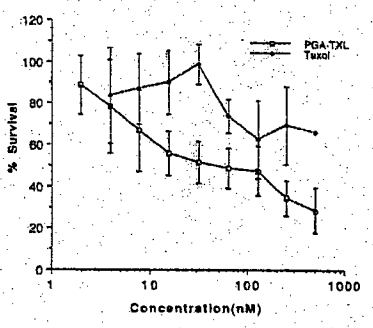
Orthotopic MDA-MB-361 tumors were established as shown in Figure 3. When the tumors had grown to at least 4-6 mm in diameter, the mice were treated with either a multiple-dose regimen of Taxol (5 or 10 mg/kg), qd7 X 3, administered i.p., or a single dose of PGA-TXL (180 mg/kg, paclitaxel equivalents), also administered i.p. Tumor diameters were measured by caliper and used to calculate volumes (l x w x w ÷ 2) that were then normalized to the starting values for each group. The volumes for the control group increased ~6.2-fold over this six-week period of observation. The volumes for the lower-dose Taxol group increased ~6.5-fold in the same period (p= not significant vs. controls), whereas the higher-dose Taxol group displayed a ~3.9-fold increase in tumor volume (p=0.1 vs. controls). The PGA-TXL group displayed a volume increase of only ~3.0-fold (p=0.0006 vs. control).

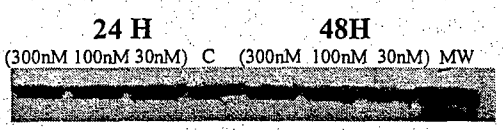
TABLE I

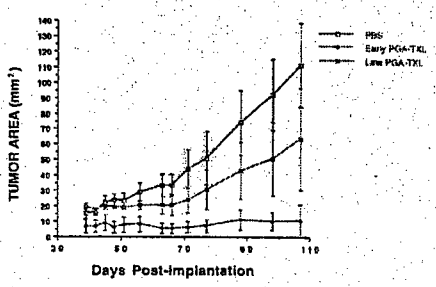
Taxol Treatment of MDA-MB-361 Human Breast Adenocarcinoma Cells Induces Early Caspase Activation and Cell Death

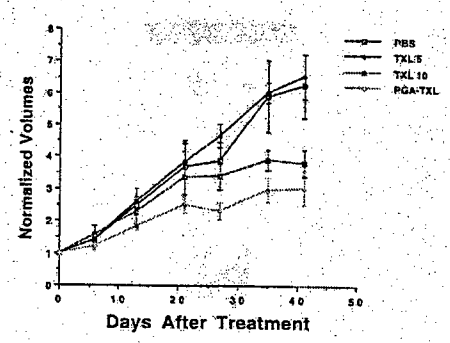
Taxol[a]	PI(+), Caspase (-)	PI(+), Caspase (+)	PI(-), Caspase (+)	Total PI(+)/Caspase(+)
0	2.0 ± 0.5[b]	0.8 ± 0.2	2.8 ± 0.6	2.8 ± 0.7/3.6 ± 0.8
2	6.3 ± 1.0	0.6 ± 0.2	2.7 ± 0.5	6.9 ± 1.1/3.4 ± 0.6
5	11.3 ± 1.5	1.2 ± 0.3	4.8 ± 0.6	12.5 ± 1.7/6.1 ± 0.8
12.5	10.6 ± 1.4	1.2 ± 0.3	5.5 ± 0.6	12.0 ± 1.6/6.7 ± 0.8
25	12.0 ± 1.5	1.7 ± 0.3	5.4 ± 0.6	13.7 ± 1.7/7.1 ± 0.8

a) nM
b) Percent of total events; mean ± SEM


PGA-TXL
Taxol
% Survival
120
100
80
60
40
20
0
1
10
100
1000
Concentration(nM)


24 H
48H
(300nM 100nM 30nM) C (300nM 100nM 30nM) MW


TUMOR AREA (mm²)
Days Post-Implantation
PBS
Early PGA-TXL
Late PGA-TXL


PBS
TXL 5
TXL 10
PGA-TXL
Normalized Volumes
0
1
2
3
4
5
6
7
8
0
10
20
30
40
50
Days After Treatment